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1. Chien, J. et al. Mol. and Cell. Endocrinology (2001) 181(1-2): 69-79
2. Chien, J. et al. Int. J. of Cancer (2001) 91(1): 46-54
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Tumor Heterogeneity and Neuroendocrine Differentiation in Non- Small Cell Lung Carcinomas; Investigation of Their Relation With Tumor Stage, P53 Protein and PcnA Expression

Özgül Sağol,¹ MD, Aydanur Kargı,¹ MD, Sülün Ermete,² MD.

Purpose: The biologic significance of tumour heterogeneity including neuroendocrine differentiation in non small cell lung carcinomas is not certain. The purpose of this study is to investigate whether there is any significant difference between non small cell carcinomas with and without tumour heterogeneity including neuroendocrine differentiation in terms of tumour stage, tumour suppressor gene alterations shown by p53 protein expression and proliferative activity shown by PCNA expression.

Methods: Paraffin sections of 57 non small cell carcinomas were reviewed and microscopic tumour heterogeneity was evaluated. The sections were all stained with neuroendocrine markers including Neuron Specific Enolase, Chromogranin A, Calcitonin and Serotonin antibodies for the evaluation of neuroendocrine differentiation, with anti-PCNA antibody for the evaluation of the proliferative activity of tumours, and with p53 antibody for the detection of mutant nuclear p53 protein expression. Standard streptavidin biotin immunoperoxidase method was used for immunohistochemical staining. Fifty seven cases were graded for cytoplasmic staining with NE markers on a scale of 0 (-), to 3 positive(+). Nuclear staining for p53 was semiquantitatively graded as follows: (-) : no staining, 1+: 1-30% of cells stained, 2+ : 30-70% of cells stained, 3+: 70% or more stained cells. Nuclear staining for PCNA was evaluated by counting 1000 tumour cells and graded as follows: 1+: 1-40% of cells stained, 2+: 40-75 % of cells stained, 3+: 75 % or more cells stained.

Results: Tumour heterogeneity was found in 12 % of non small cell lung carcinomas on routine sections. Tumours with and without heterogenous areas did not show statistically significant difference in terms of tumour

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¹Dokuz Eylül University Faculty of Medicine, Department of Pathology, İzmir-Türkiye

²Education Hospital for Chest Diseases and Surgery, Tepecik, İzmir-Türkiye

stage, proliferative activity and p53 expression. five percent of 57 non-small cell carcinomas reacted with two neuroendocrine markers. 42 % of cases were positive for only one neuroendocrine marker. There was no statistically significant difference between the cases which were negative and cases which were positive with 1 or 2 neuroendocrine markers, in terms of proliferative activity and p53 expression.

Seventy-one percent of 57 non small cell carcinomas reacted with p53 antibody. Cases with p53 protein expression were not different from negative cases in terms of proliferative activity, neuroendocrine differentiation and tumour stage. All cases of non small cell carcinomas reacted with PCNA antibody with the mean staining index of 61.6 %. PCNA staining indices did not show statistically significant difference between subtypes and tumour stages.

Conclusion: A complete NE differentiation demonstrated by positivity for multiple NE markers similar to NE tumours is quite unusual. Heterogeneity and neuroendocrine differentiation in NSCLCs shown by light microscopy or by one or two NE marker positivity does not correlate with tumours stage which is the most valuable predictor of prognosis and with other factors like p53 and PCNA reactivity whose influence on prognosis is debatable. *Ann Med Sci* 1999;8:14-21

Key words: Non small cell lung cancer, tumour heterogeneity, neuroendocrine differentiation, p53, PCNA.

Microscopic tumour heterogeneity defined as the presence of different histologic types within the same tumour is reported in 13-63% of lung cancers.^{1,2,3} As a practical manner most lung cancers can be classified as either I- non-small cell lung cancers (NSCLC) including squamous cell carcinoma, adenocarcinoma, adenosquamous carcinoma and large cell carcinoma or II- small cell lung cancers (SCLC).^{1,4,5} While the presence of small cell carcinoma component in NSCLC, regardless of the extent of it, implies poor prognosis and different treatment, the clinical significance of coexistence of different types of NSCLCs is unclear.⁶

Neuroendocrine (NE) differentiation detected by light microscopy or immunohistochemistry has been reported in 10-50 % of NSCLCs.^{4,5,7,8} Neuroendocrine differentiation is most commonly reported in large cell carcinomas and poorly differentiated adenocarcinomas.^{5,7,9,10} Recent studies have shown conflicting results regarding both the criteria for definition and the clinical significance of NE differentiation.^{5,9}

Current advances in molecular biology revealed that mutations of the P53 gene are one of the most frequent genetic abnormalities in human neoplasms including lung cancer.^{11,12} P53 gene mutations were reported in 37-80 % of all types of lung cancer.^{13,16} In some studies mutation of p53 gene has been reported to be associated with poor prognosis, while in others conflicting results have been reported.^{5,13,17-25} There are also confusing results on the relation of mutant p53 protein expression with the stage of

the disease.^{5,26,27} Besides, it has been suggested in some reports that the mutant p53 gene enhances proliferative activity by inducing PCNA gene and that the p53 protein expression increases with the degree of neuroendocrine differentiation in NE carcinomas.^{13,28}

Proliferative activity expressed by PCNA expression was reported to be high in lung carcinomas.²⁹ The prognostic value of PCNA expression in lung cancer is not clear.³⁰⁻³²

The aim of this study is to investigate the microscopic heterogeneity and NE differentiation in NSCLC as it was shown by immunohistochemical demonstration of NE markers and to search for any difference in p53 tumour suppressor gene mutation, proliferative activity and tumour stage between NE marker positive and negative tumors. Besides, the relation of p53 expression and proliferative activity of tumours were evaluated.

Materials and Methods

Formalin fixed and paraffin embedded archival material of 57 lung carcinomas diagnosed in our department between the years 1991 and 1995 were included in this study. Pathologic material was obtained by endoscopic biopsy in 24 of cases, and by lobectomy in 33 cases. The cases were staged according to TNM system.³³

Eight to 10 Haematoxylin and Eosin (H&E) sections from each tumour excised by lobectomy

and endoscopic biopsy materials were screened to assess histologic subtype according to WHO classification.³⁴ Tumour heterogeneity defined as the presence of different histologic types within the same tumour was evaluated in each tumour. Five micron sections from selected representative paraffin blocks were taken on poly-l-lysine coated slides. Paraffin sections were stained with NE markers including Neuron Specific Enolase (NSE), (DakoH14, 1/100 dilution), Chromogranin A (Dako Kr A, Dak-A3, 1/100 dilution), Calcitonin (Dako, 1/200 dilution), Serotonin (Dako-5HT- H209, 1/10 dilution) for the evaluation of NE differentiation. The sections were stained with anti-PCNA antibody (Dako, 1/100 dilution) for the evaluation of proliferative activity of tumours, and with p53 antibody (Dako, DO7, 1/50 dilution) for detection of mutant nuclear p53 protein. The streptavidin biotin immunoperoxidase method was used for immunohistochemical staining. A microwave antigen recovery technique requiring the boiling of the deparaffinised sections in 0.01 M citrate was used prior to immunostaining. Diaminobenzidine was used as a chromogen. Carcinoid tumour of the lung known to express all NE markers used in this study and a breast carcinoma section known to express p53 protein and PCNA antibodies were used as positive controls. Negative controls were provided by omitting of primary antibody during the staining procedure.

Fifty seven cases were graded for cytoplasmic staining with NE markers on a scale of 0 (-), to 3 positive(+). (-) indicated no staining, 1+, rare scattered individual cells with positively stained cytoplasm, 2+, small clusters of stained cells and 3+, extensive staining. Nuclear staining for p53 was semiquantitatively graded as follows: (-): no staining, 1+: 1-30% of cells stained, 2+:

30-70% of cells stained, 3+: 70% or more stained cells.

Nuclear staining for PCNA was evaluated by counting 1000 tumour cells and graded as follows: 1+: 1-40% of cells stained, 2+: 40-75% of cells stained, 3+: 75% or more cells stained.

The results of this study were evaluated statistically by using Rank correlation, Kruskal Wallis, Kolmogorov Smirnov and Chi-Square tests.

Results

Ages of the 57 patients with NSCLC ranged from 34 to 77 (mean: 55.5) years. Six of the patients were females and 51 were males. According to the TNM staging system, 18 cases were in stage 1, 6 cases were in stage 2, 7 were in stage 3 A, 10 were in stage 3 B and 16 cases were in stage 4. According to the WHO classification³⁴, 38 of cases were squamous cell carcinomas²² well differentiated, 4 moderately differentiated and 12 poorly differentiated, 14 were adenocarcinomas (10 well differentiated, 2 moderately differentiated and 2 poorly differentiated), 2 were adenosquamous carcinomas and 3 were large cell carcinomas. 7 (12.28%) of 57 NSCLC cases showed tumour heterogeneity. Two of these cases were adenosquamous carcinomas and 5 were squamous cell carcinomas with focal adenoid structures. Tumour heterogeneity was found in only resection materials (19.4%). Table 1 shows the distribution of tumour stage, p53 expression and PCNA positivity in heterogenous and nonheterogenous NSCLCs. Table 2 shows staining with NE markers, p53 and PCNA antibodies and stages of the cases.

Table 1. p53 and PCNA expression in heterogenous and non heterogenous NSCLCs

	Stage					PCNA score*			P53 score**			
	1	2	3a	3b	4	1	2	3	0	1	2	3
Heterogenous (n:7)	4	1	1	1	0	1	3	3	2	2	0	3
Pure (n:50)	14	4	7	9	16	13	13	22	14	5	10	19

* Two cases were insufficient for PCNA staining.

** Two cases were insufficient for P53 staining.

Table 2. Stages, p53 and PCNA scores and NE marker positivity in NSCLCs

Number of positive NE markers	Stage					P53 scores*				PCNA scores**			Total
	1	2	3a	3b	4	0	1	2	3	1	2	3	
0	11	3	5	4	7	8	5	7	10	7	9	14	30
1	7	2	2	5	8	7	1	3	11	6	6	10	24
2	0	1	0	1	1	1	1	0	1	1	1	1	3

* 2 cases are insufficient for P53 staining.

** 2 cases are insufficient for PCNA staining.

Table 3. PCNA staining indices for NSCLCs

N:55	PCNA staining indices (% of positive stained nuclei)
Minimum	3.2
Maximum	99.2
mean	61.65
Median	64.00
Standard deviation	23.84

Thirty (52.63%) of 57 NSCLC s showed no staining for any of the NE markers. Twenty-four (42.1%) cases were stained for only 1 NE marker. (2 of the cases were stained with only calcitonin, 22 were stained with only NSE) (28%=+, 56%=2+, 16%=3+). Two squamous cell carcinomas and 1 adenosquamous carcinomas (5.26%) showed staining with both NSE and calcitonin. None of our cases were stained with antibodies against serotonin or chromogranin. Thirty-nine (70.91) % of 57 NSCLCs were stained with P53 antibody. Two of the cases were found to be insufficient for evaluation. Fifty-five of 57 (96%) cases were stained positive with PCNA antibody. Again, 2 cases were found to be insufficient for evaluation. PCNA staining indices are shown in table 3.

As a result no statistically significant difference was found between the heterogenous and the non heterogenous tumours in terms of tumour stage, mutant p53 protein expression and proliferative activity. There was also no statistically significant difference between tumours with no NE marker positivity and tumours stained with 2 NE markers or with 1 marker only, in terms of tumour stage, p53 expression and proliferative activity. P53 positive and negative cases showed no difference in terms of tumour stage. No relation was found between the scores of staining and stage of tumour in p53 staining. There were also no relation between PCNA indices, tumour stage and histologic type as well as between tumours with high PCNA indices and tumours with low indices in terms of p53 scores.

Discussion

The histopathologic heterogeneity has been reported within an individual tumour in up to 10-63 % of sufficiently sampled lung cancers by light microscopy alone. In some studies, non-small cell lung cancer heterogeneity was found to correlate with tumour stage^{1,2,3}. In this study, microscopic heterogeneity was detected in 12.8% of NSCLCs and heterogenous tumours did not show statistically significant difference in terms of tumour stage, proliferative activity, p53 mu-

tations and NE differentiation suggesting that

the dominant pattern determines the tumor behaviour in NSCLCs. Our finding of heterogeneity by light microscopy in only resection materials, demonstrates the need for sufficient material in order to recognize tumour heterogeneity.

In addition, up to half of NSCLCs which by light microscopy have typical features of adenocarcinoma, squamous cell carcinoma or large cell carcinoma and lack light microscopic features of NE differentiation may demonstrate focal or more extensive positivity for a NE marker^{4,5,7-10,35}. NE differentiation was found in 3- 50% of NSCLCs^{5,7,8,36}. NE differentiation are most commonly reported in large cell and squamous cell carcinomas^{5,7,9,10}. NSE, Chromogranin, Serotonin, Calcitonin, Synaphtophysin and Leu7 are the most commonly used immunohistochemical NE markers to reveal neuroendocrine features. Among the NE markers used in this study, NSE is an isoenzyme of a glycolytic enzyme enolase which is found as dimers of alpha, beta and gamma subunits. Gamma-gamma dimers are thought to be specific for neural tissues although in some recent reports it's claimed that this dimer can be found in non neural tissues³⁷. Chromogranin is an acidic protein found in cytoplasmic granules. The role of chromogranins is uncertain although there is some evidence that they take part in the maturation of granules, regulation of enzyme activity of granules and peptide hormone synthesis³⁸⁻³⁹. Calcitonin and serotonin are specific products secreted by NE cells⁷. Whether positivity with only one of these markers represents NE differentiation or not, is debatable.

In many studies, NSE positivity alone in NSCLCs was reported to be nonspecific and insufficient for indicating NE differentiation. In some studies positivity with only one marker other than NSE was thought to be sufficient to indicate NE differentiation.⁴⁰ Linnolia et al used 9 NE markers in their study and regarded the tumours which were positive for 4 or more NE markers as tumours with NE differentiation. In the latter study it is believed that tumours which are indistinguishable from NE tumours immunohistochemically should be called tumours with NE differentiation⁷. In the study of Brambilla et al, 3 NE marker positivity was accepted to be sufficient to indicate NE differentiation⁹. On the other hand, there are studies accepting 2 or more marker positivity^{4,9}. In an electron microscopic (EM) study of Wilson et al, it was shown that all of the tumours with dense core granules detected by EM, showed immunohistochemically positive reaction with NSE, while others without dense core granules failed to react, indicating that NSE might indeed rep-

resent NE differentiation⁸. It has been suggested that NSE detects cells with minimal NE differentiation (ie. those with few secretory granules or low levels of hormones) that can not be detected by other techniques¹¹.

In any case, the clinical significance of NE differentiation either shown by NSE positivity alone or a combination of markers is a subject of

controversy. In some studies, it has been claimed that, NSE immunoreactivity correlated with improved survival⁹. Graziano et al and Scov et al reported that NSCLCs with NSE positivity showed increased response to chemotherapy⁹. However in some other studies, no such relation had been found between NE differentiation and tumours stage, relapse and survival in NSCLCs.



Figure 1. Cytoplasmic staining with calcitonin in adenosquamous carcinoma (X100)

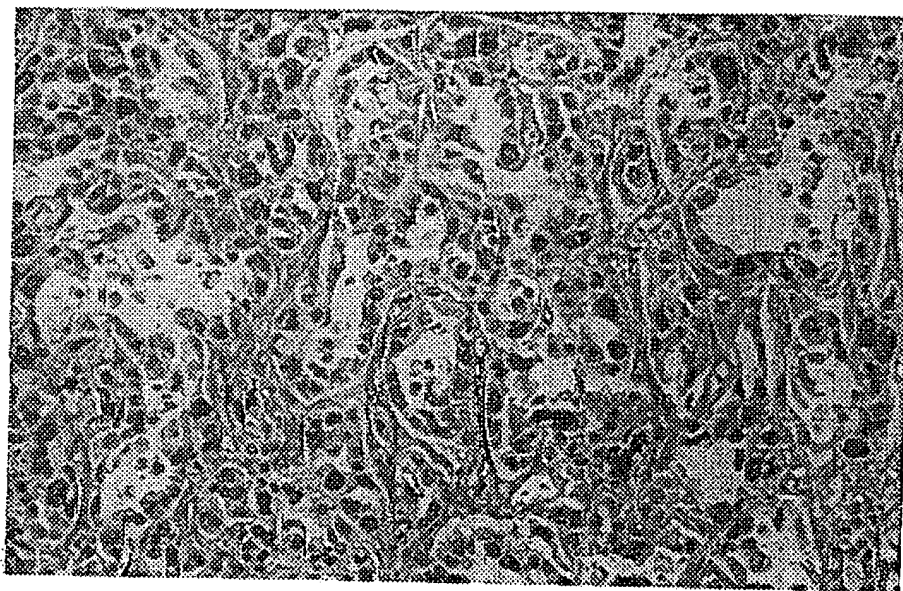


Figure 2. Nuclear staining with P53 antibody in adenocarcinoma (X100)

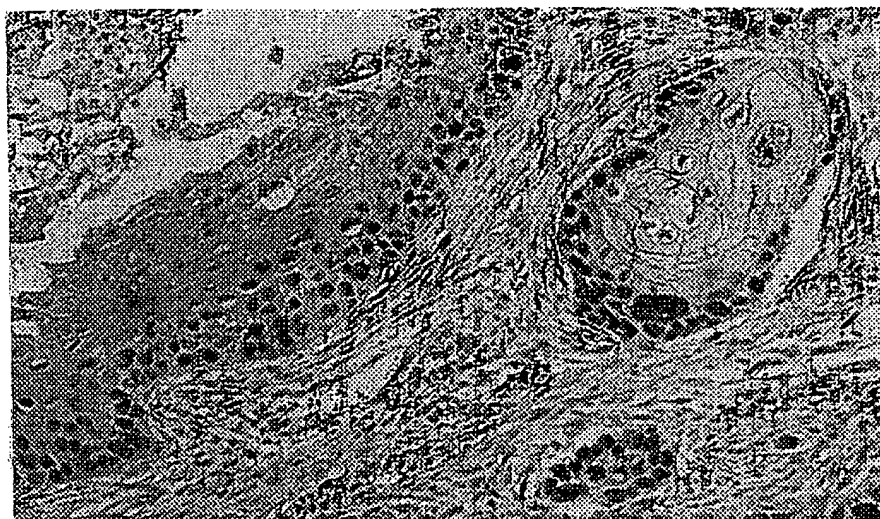


Figure 3. Nuclear staining with PCNA antibody in a squamous cell carcinoma. (X100)

In the present study, there are only three cases immunoreactive for more than one marker. On the other hand the finding of NSE positivity in 43,8% (25 of 57 cases) of cases is in accordance with previous reports. We have found no statistically significant difference between stages of NSCLCs with and without NSE immunoreactivity in the tumour tissues.

The heterogeneity of lung cancers, including NE differentiation in NSCLCs is currently being explained by an hypothesis that all lung cancers are ultimately derived from a common stem cell which in addition to undergoing malignant transformation, can potentially differentiate into any of the different cell types or into combined types³. It's also believed that the phenotype of the lung cancer, including not only the cell types but other characteristics such as growth rate, metastatic potential and response to therapy represents the expression of the specific genetic abnormalities. Abnormalities of tumour suppressor gene P53, ranging from complete deletion to point mutation, constitute some of the most frequently encountered genetic defects in human cancer including lung cancer^{20,23}.

P53 is a tumour suppressor gene encoding 53 kD intranuclear protein which is known to regulate transcription, DNA replication and induces apoptosis. This protein can not be detected by immunohistochemistry in normal conditions but mutations of p53 gene stabilises the p53 protein and makes it accumulate in the nucleus and be detected by immunohistochemistry^{22,42-44}. Mutations in p53 gene is detected in 37 to 80% of all lung carcinomas and there seems to be no predilection of p53 mutation for any specific phenotype^{13,16,19,26,45}. In the present study, 70.91% of NSCLC cases reacted with p53 antibody immunohistochemically. Unlike some studies sug-

gesting that mutations appear in early stages of the disease, p53 positivity and the degree of the staining did not vary between different stages indicating that mutations can appear in every stage of the disease^{5,46,47}.

There are only a few studies investigating the relation between p53 mutation and NE differentiation. Barbareski et al. reported that with decreasing NE differentiation from carcinoid to small cell carcinoma, the frequency of p53 mutation and PCNA activity is increased^{13,26}. On the basis of such observations, it can be speculated that p53 mutation might be responsible for loss of NE features while contributing malignant transformation. If that is the case, than p53 mutation in NSCLC s with NE differentiation could be expected to be less frequent than the ones without NE differentiation. In order to investigate the validity of this suggestion, we compared the frequency of p53 mutations and proliferative activity in NE marker positive and negative NSCLCs. Our results did not show any statistically significant difference in p53 mutation or proliferative activity between the NE marker positive (one or two marker positivity) and negative cases. One reason for these findings might be minimal NE differentiation in our cases, most of which had had only NSE positivity. It might be more meaningful to compare the p53 expression of NSCLCs or SCLCs with varying degrees of NE differentiation to test the validity of this hypothesis.

Proliferative indices in our cases are consistent with the study of Theunissen et al, indicating that the proliferative activity is high in lung carcinomas²⁹. In our study no statistically significant relation could be found between the proliferative indices and tumour stage and histologic subtypes. Lee et al also found no relation

between PCNA indices and tumour stage while Ishida et al found statistically significant relation⁴⁸. Haerslev et al, found that, 52% of the lung tumours included in their study had high proliferative activity and like Ebina et al, they found positive correlation between p53 mutations and PCNA positivity^{16,18}. Korkolopoulou et al also found positive correlation between p53 expression and PCNA indices¹³. They indicate that wild type p53 gene selectively suppresses the PCNA mRNA and PCNA protein in the nucleus and that, mutant p53 gene has an inducing role on p53 gene¹³. Fontanini et al, did not find any relation between p53 gene and PCNA expression⁴⁹. In our study in consistence with the latter study no correlation has been detected between PCNA indices and p53 protein expression.

In conclusion, although NSE positivity which may reflect minimal NE differentiation, is not unusual in NSCLCs. It appears that a complete NE differentiation demonstrated by positivity for multiple NE markers similar to NE tumours is quite unusual. The genetic basis for such a differentiation remains to be investigated. Heterogeneity and neuroendocrine differentiation in NSCLCs shown by light microscopy or by one or two NE marker positivity does not correlate with tumours stage which is the most valuable predictor of prognosis and with other factors like p53 and PCNA reactivity whose influence on prognosis is debatable.

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